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Hypoxia-induced acidification causes mitoxantrone resistance not mediated by drug transporters in human breast cancer cells

A.E. Greijer [a,*], M.C. de Jong [a], G.L. Scheffer [a], A. Shvarts [b], P.J. van Diest [b] and E. van der Wall [b]
[a] *Departments of Pathology and Medical Oncology, VU University Medical Centre, Amsterdam, The Netherlands*
[b] *Departments of Pathology and Division of Internal Medicine and Dermatology, UMCU, Utrecht, The Netherlands*

Abstract. Hypoxia has clinically been associated with resistance to chemotherapy. The aim of this study was to investigate whether hypoxia induces resistance to doxorubicin and mitoxantrone, two common drugs in cancer treatment, in MCF-7 breast cancer cells, and SW1573 non-small lung cancer cells. In addition, the role of drug transporters P-gp, BCRP and MRP1 was analysed. Hypoxia induced resistance in MCF-7 cells to mitoxantrone shifted the IC_{50} value from 0.09 μM ($\pm$0.01) to 0.54 μM ($\pm$0.06) under hypoxia, whereas survival of MCF-7 and SW1573 cells in the presence of doxorubicin was not altered. Accumulation of mitoxantrone and daunorubicin, a doxorubicin fluorescent homologue, appeared to be 5.3 and 3.2 times lower in MCF-7 cells, respectively. Cytotoxicity assays showed no increased functionality of the drug transporters P-gp, BCRP and MRP1 under hypoxia. In addition, protein levels of these drug transporters were not changed. Medium of the MCF-7 cells became more acidic under hypoxia thereby causing a decreased uptake of mitoxantrone. Hypoxia induces mitoxantrone resistance in MCF-7 cells not mediated by the three major MDR transporters. Hypoxia-induced acidification may cause this resistance by decreased cellular uptake together with a lowered cytotoxicity due to pH-dependent topoisomerase type II activity.

Keywords: Acidification, hypoxia, doxorubicin, mitoxantrone, multiple drugs resistance

1. Introduction

Drug resistance is a common phenomenon in cancer patients. Resistance can be acquired after initial response to chemotherapy, or cancers can be resistant from the start. Resistance to chemotherapy may be due to a number of factors. Causes of resistance include a low local drug concentration in the tumour due to insufficient drug delivery by decreased density of blood vessels, a low cellular uptake, or high efflux by overexpression of drug transporters. In addition, intracellular mechanisms like tumour growth kinetics and structural or metabolic adaptations may result in drug resistance [5,24]. The microenvironment of cells add to the drugs resistance, e.g. hypoxia may induce resistance by selecting for cells with diminished capability of apoptosis [10].

*Corresponding author: Astrid E. Greijer, PhD, Department of Pathology, VU University Medical Centre, PO Box 7057, 1007 MB Amsterdam, The Netherlands. Tel.: +31 20 4444049; Fax: +31 20 4442964; E-mail: ae.greijer@vumc.nl.

Hypoxia is often observed in solid tumours [11]. Clinical observations have revealed that tumour hypoxia is related with increased resistance to radiotherapy and chemotherapy [3,8], and thereby with poor prognosis [2]. The mechanisms by which hypoxia results in chemoresistance are largely unknown. Tumour hypoxia may develop in tumours with a high growth rate relative to the capacity of the microvasculature to supply nutrients and oxygen, causing cell cycle disruption or arrest, DNA overreplication and induction of stress proteins as reviewed by Teicher [24].

In these tumours the key regulator induced by hypoxia, hypoxia inducible factor 1 (HIF-1), is overexpressed. HIF-1 is a transcription factor consisting of α and β subunits, of which the α subunit is regulated by oxygen deprivation. In a hypoxic microenvironment, the α subunit is stabilized, complexes to HIF-1β and becomes transcriptionally active. Different classes of genes are regulated by HIF-1 including glycolytic enzymes to allow adaptation to anaerobic glucose metabolism [12]. The switch to anaerobic glycolysis results in a more acid microenvironment. This is

often observed in tumour cells, even in the presence of sufficient oxygen levels (Warburg effect) [26].

Potential explanations for hypoxia-induced chemoresistance include a rapid efflux of intracellular drugs by upregulation of drug transporters. In a multicellular tumour model of spheroids using a human epidermoid carcinoma epithelium cell line, the MDR transporter P-glycoprotein (P-gp) was indeed upregulated in regions of hypoxia and low levels of reactive oxygen species. HIF-1α was upregulated in areas where P-gp expression was increased [27]. Interestingly, the promoter region of the MDR1 gene, encoding P-gp, harbours a hypoxia responsive element (HRE) to which HIF-1 binds [4]. The increased expression of P-gp under hypoxia resulted in a mild level of resistance to the drug doxorubicin as analysed in survival assays [4].

Further explanations for hypoxia-induced chemoresistance may be an acid environment in hypoxic regions of tumours. Lower pH influences passive cellular uptake of several cytostatics, such as doxorubicin and mitoxantrone. These two anthracyclines, commonly used for treatment of breast cancer, become positively charged in acid environments, which decreases their passive translocation over the cellular membranes [16].

The aim of this study was to study whether hypoxia gives rise to resistance to doxorubicin and mitoxantrone in breast cancer cell lines and whether this is related to expression of drug transporter proteins. In addition, the influence of pH of the cellular microenvironment in acquiring drug resistance was analysed.

2. Materials and methods

2.1. Cell culture

A mitoxantrone resistant breast cancer cell line overexpressing breast cancer resistance protein (BCRP), MCF-7 MR [20], a doxorubicin resistant variant overexpressing P-gp, MCF- 7 Dox40 [23], and the parental, sensitive, MCF-7 were used. SW-1573 and the MRP1 overexpressing cell line, SW1573 2R120, have been described previously [15]. MCF-7 MR and MCF-7 Dox40 were cultured in RPMI supplemented with 10% foetal calf serum (FCS). SW1573 and SW1573 2R120 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS. The cell lines with a resistant phenotype, MCF-7 Dox40, MCF-7 MR, and SW1573 2R120 were cultured in the presence of 4 nM doxorubicin and 8 nM mitoxantrone, respectively, until 3–10 days before experiments.

2.2. Exposure to hypoxia

Cells were cultured under hypoxia as described previously in a hypoxia chamber (Billups-Rothenberg, Del Mer, CA, USA) flushed with premixed gas consisting of 1% O_2, 5% CO_2, and 94% N_2 during 4 minutes [25]. After 1 hour, flushing was repeated for 2 minutes.

2.3. Cytotoxicity assays

Exponentially growing cells were seeded in triplicate in 96-wells plates (number of cells per well: MCF-7, MCF-7 MR, MCF-7 D40: 5,000; SW1573, SW1573 2R120: 6,000) and were cultured in the presence of serial dilutions (ranging from 0.001 μM to 100 μM) of doxorubicin (Farmitalia Carlo Erba, Brussels, Belgium) or mitoxantrone (AHP Pharma BV, Hoofddorp, The Netherlands). Ten μM verapamil (Sigma Chemical Co, St Louis, MO USA), 100 ng Ko143 (a kind gift from Dr. J.D. Allen [1]), or probenecid (Sigma Chemical Co) were added as inhibitors of P-gp1, BCRP and MRP1, respectively. Subsequently, cells were cultured for 72 hours under normoxia and hypoxia, after which cell survival was determined using sulphorhodamin B (SRB) [6]. Colour intensity was measured at 540 nm with a SpectraFluor (Tecan Benelux, Giesen, The Netherlands). Experiments were repeated three times. The drug concentration which produced a 50% inhibition of growth (IC_{50}) was calculated by linear regression analysis of the linear portion of dose response curves. The resistance factor (RF) was calculated as the ratio of the IC_{50} of cells under normoxia without inhibitor to the IC_{50} of the experimental samples.

2.4. Drug accumulation study

For determination of doxorubicin and mitoxantrone accumulation under normoxia and hypoxia, intracellular drug concentrations were measured by FACS analysis. In order to mimic drug uptake under hypoxia, medium was collected from MCF-7 cells cultured for 24 hours under hypoxia. Thereby, not only the influence of hypoxia, but also hypoxia-induced acidification of medium was studied. MCF-7 cells were exposed to 20 μM of the fluorescent doxorubicin analogue daunorubicin, or 20 μM mitoxantrone for 1 hour. Cells were then washed twice with ice-cold phosphate buffed saline (PBS) and analysed by FACSstar (Becton Dickinson and Company, Oxnard, CA, USA).

2.5. Immunoblot analysis for drug transporters

Western blotting was done by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cells were incubated during 24 hours under normoxia and hypoxia, after which cells were washed with phosphate buffered saline (PBS) and directly lysed in Leammli buffer and 10^5 cell equivalents were loaded onto a 8% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes (Hybond, Amersham, Arlington Heights, IL, USA). Membranes were blocked in 3% non-fat milk powder dissolved in PBS with 0.05% Tween-20 (PBS-T) and incubated for 1 hour with a mouse monoclonal antibody against HIF-1α (BD Transduction Laboratories, Lexington, KY USA) diluted 500 fold or overnight with the mouse monoclonal BXP-21 [17] against BCRP (1 : 400), the JSB-1 [22] mouse monoclonal against P-gp (1 : 200), or the rat antibody MRPr1 [21] against MRP1 (1 : 500). As control, the blot was stained with a monoclonal against α-tubulin in a 1 : 1000 dilution (Amersham, Buckingham, UK) for 1 hour at rt. After washing, membranes were incubated with the appropriate secondary antibody conjugated with horseradish-peroxidase. Chemoluminescent detection of the antibody binding was performed using the ECL plus system (Amersham Biosciences, Piscataway, NJ, USA).

2.6. Statistical analysis

The non-parametric Mann–Whitney test for independent samples (SPSS for Windows version 9.01, 1999 SPSS Inc, Chicago, IL, USA) was used to compare IC_{50} values between normoxic and hypoxic samples between different cell lines.

3. Results

3.1. Hypoxia induces resistance to mitoxantrone, but not to doxorubicin in MCF-7 cells

MCF-7 cells were cultured for 72 hours with varying concentrations of doxorubicin or mitoxantrone under normoxia (20% O_2) or hypoxia (1% O_2). The relative resistance factor of cells cultured under these different conditions are presented in Fig. 1. Hypoxia had no effect on the cytotoxicity of doxorubicin in MCF-7 cells. However, hypoxia shifted the IC_{50} value of mitoxantrone cytotoxicity from MCF-7 cells significantly upwards from 0.09 μM ($\pm$0.01) to 0.54 μM ($\pm$0.06) (Table 1), which indicates a decrease in cytotoxicity of mitoxantrone with a factor 6.0 for these cells under hypoxic conditions.

3.2. Hypoxia does not increase functionality of MDR transporters

To evaluate whether the activity of multi-drug-resistance (MDRs) pumps was altered under hypoxia, cells overexpressing P-gp, BCRP, and MRP1 were used. First, reduced cytotoxicity of mitoxantrone under hypoxia was studied in BCRP-overexpressing cell line MCF-7 MR and by the use of specific BCRP inhibitor, Ko143 (Fig. 1A). Hypoxia-induced mitoxantrone resistance was not observed in MCF-7 MR cells. When the BCRP transporter inhibitor Ko143 was added, sensitivity to mitoxantrone was restored in MCF-7 MR cells. However, decreased mitoxantrone cytotoxicity in MCF-7 cells observed under hypoxia was not affected by Ko143. This suggests that hypoxia-induced resistance to mitoxantrone is not caused by increased functionality of BCRP (Fig. 1A).

Incubation of tumour cells with doxorubicin for 72 hours under hypoxic conditions did also not alter activities of P-gp as verified in MCF-7 cells and P-gp-overexpressing MCF-7 Dox40 cells with the P-gp blocking agent verapamil (Fig. 1B). These data are in line with the fact that doxorubicin sensitivity was not affected by hypoxia. Since beside P-gp and BCRP, MRP1 is a commonly expressed transporter, SW1573 and the MRP1 overexpressing SW1573 2R120 cells were analysed under hypoxia. Similar to P-gp and BCRP, activity of the MRP1 pump was not affected by hypoxia (Fig. 1C). The unaltered functionality of MRP1 was verified in SW1573 and MRP1-overexpressing SW1573 2R120 cells using a specific MRP1 inhibitor, probenecid.

In agreement with the functionality data of P-gp, BCRP, and MRP1, protein levels of these MDR transporters were not changed under hypoxia (Fig. 2). The level of BCRP expression was identical under normoxia and hypoxia in the MCF-7 as well as in the BCRP-overexpressing cell line. MDR1 encoded protein could only be detected in the P-gp-overexpressing MCF-7 Dox40 and in the MRP1 overexpressing SW1573 2R120 cell lines. In the sensitive MCF-7 and SW1573 cell lines MDR expression under normoxia as well as under hypoxia was below the detection limit. No difference was observed in P-gp and MRP1 expression under hypoxia.

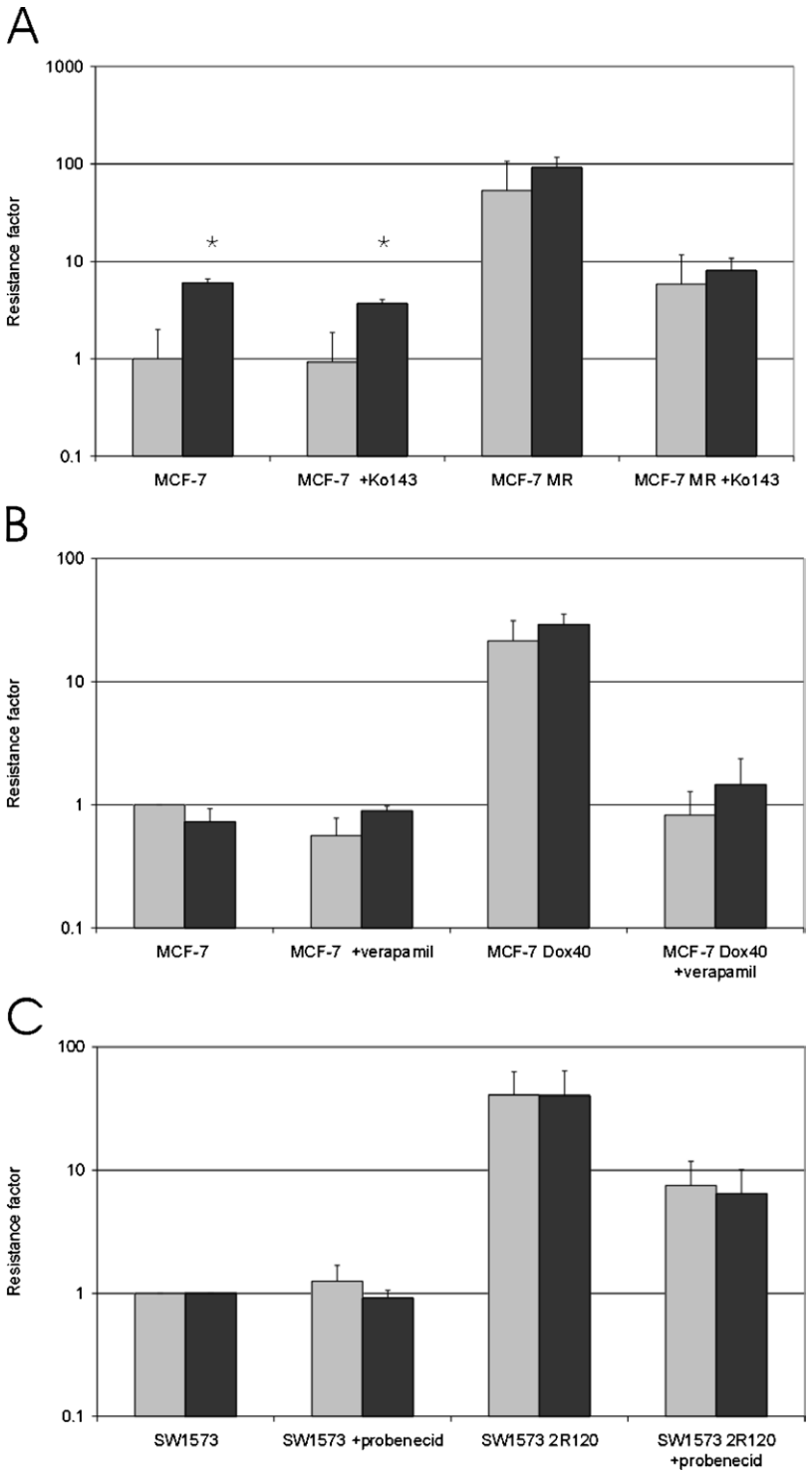

Fig. 1. Resistance factors of cells cultured with doxorubicin or mitoxantrone under normoxia and hypoxia. A: MCF-7 and MCF-7 MR, overexpressing BCRP, in presence of mitoxantrone, B: MCF-7 and MCF-7 Dox40, overexpressing P-gp, in presence of doxorubicin, C: SW1573 and SW1573 2R120, overexpressing MRP1, in presence of doxorubicine. Gray boxes: normoxia, dark boxes: hypoxia. Results shown are means $\pm$ SD of three experiments. Asterisk indicates significant differences ($P \leqslant 0.05$).

Table 1
IC_{50} values of MCF-7 cultured in presence of doxorubicin or mitoxantrone under normoxia and hypoxia. Experiments were performed in triplicate

Cell line	Drug	Normoxia		Hypoxia		Significance
		IC_{50}	SD	IC_{50}	SD	
MCF-7	doxorubicin	0.21	0.10	0.30	0.20	$P > 0.05$
MCF-7	mitoxantrone	0.090	0.01	0.54	0.06	$P = 0.05$

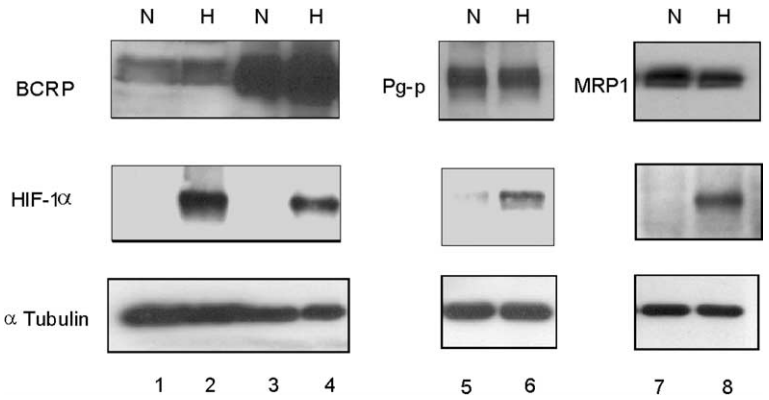

Fig. 2. Western blot analysis of protein levels of MDR1, BCRP, MRP1 and as control for hypoxia HIF-1α. α-tubulin is used as loading control. Cells were incubated under hypoxia and normoxia during 72 hours, after which cells were lysed and MDR expression was analysed. Lane 1: MCF-7 under normoxia, 2: MCF-7 under hypoxia, 3: MCF-7 MR, overexpressing BCRP, under normoxia, 4: MCF-7 MR under hypoxia, 5: MCF-7 DOX40 overexpressing P-gp, under normoxia, 6: MCF-7 DOX40 under hypoxia, 7: SW1573 2R120, overexpressing MRP1, under normoxia, 8: SW1573 2R120 under hypoxia.

3.3. Reduced accumulation of mitoxantrone under hypoxia

To evaluate whether lower toxicity of mitoxantrone under hypoxia in MCF-7 cells might be due to a lower drug uptake resulting in a decreased intracellular drug concentration, mitoxantrone and daunorubicin concentrations in MCF-7 cells were measured by FACS analysis. The homologue of doxorubicin, daunorubicin was used, since the latter has a higher fluorescent property. Uptake of both mitoxantrone and daunorubicin in MCF-7 cells was decreased during hypoxia (Fig. 3). Uptake of mitoxantrone was more reduced under hypoxia (factor 5.3) compared to daunorubicin (factor 3.2).

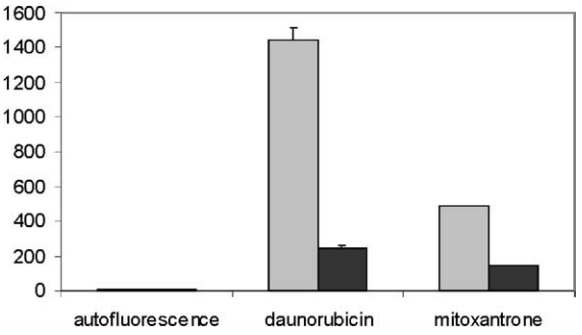

Fig. 3. Drug accumulation of daunorubicin and mitoxantrone in MCF-7 cells under normoxia and hypoxia. MCF-7 cells were exposed to 20 μM of the fluorescent doxorubicin analogue daunorubicin, or 20 μM mitoxantrone for 1 hour. Results shown are means $\pm$ SD of two experiments. Grey boxes: normoxia, dark boxes: hypoxia.

3.4. Acidic pH reduces uptake of mitoxantrone and daunorubicin

For analysing the influence of hypoxia on the passive uptake of mitoxantrone and daunorubicin, conditioned medium of cells incubated under hypoxia was used from MCF-7 cells cultured under hypoxia for 24 hours. Therefore not only hypoxia, but also hypoxia-induced acidification of medium was analysed. Medium of hypoxic samples had a pH of 6.85 compared to 7.12 of medium under normoxia.

4. Discussion

In this study, the influence of hypoxia on resistance to chemotherapy was evaluated in cancer cell lines.

Under hypoxia, MCF-7 breast cancer cells appeared to be more resistant (6 fold) to mitoxantrone compared to normoxia. Survival of MCF-7 and SW1573 cell lines in presence of doxorubicin was not altered under hypoxia. Functionality of the three most important MDR transporters, P-gp, BCRP and MRP1, was unchanged when cells were incubated under 1% oxygen during 72 hours. This is in contrast to studies in multilayer spheroids of the human epidermoid carcinoma cell line from the mouth (strain KB) that showed a mild resistance to doxorubicin under hypoxia [4]. Since the spheroids were incubated at 1% oxygen the centre of the spheroids may suffer from lower oxygen levels than cells in monolayers. In addition, the use of spheroids instead of cell monolayers may give rise to other factors influencing resistance to doxorubicin, like decreased drug delivery.

Resistance to mitoxantrone could not be explained by upregulation of drug transporters, a common cause of multidrug resistance. Mitoxantrone is rapidly pumped out of cells by membrane bound BCRP [7], but inhibition of this transporter by Ko143 did not reverse resistance to mitoxantrone under hypoxia. Hypoxia-induced resistance to mitoxantrone was less pronounced in cells overexpressing BCRP which may be caused by the lower intracellular drug concentration due to the high levels of BCRP. Furthermore, BCRP protein levels were not altered under hypoxia.

As mitoxantrone intracellular drug concentrations were 5.2 times lower at hypoxia, decreased cytotoxicity under hypoxia to mitoxantrone may well be due to a lower intracellular drug concentration. This is supported by the fact that passive transport through the cellular membrane of anthracyclines such as doxorubicin and mitoxantrone is influenced by the degree of acidity of the microenvironment [13,14]. Low extracellular pH can reduce cytotoxicity by a direct effect of ion gradients on drug distribution and ion trapping [18]. Ion trapping occurs when anthracyclines, which are weakly basic drugs, will concentrate in more acidic compartments. Therefore, ion trapping results in hindering anthracyclines to reach their intracellular target. Since hypoxia induces a more acidic environment by increased production of lactate caused by anaerobic glycolysis, this may hamper passive influx of mitoxantrone into cells. In addition, cytotoxicity of mitoxantrone at pH 6.8 is decreased compared to a pH 7.4. Cytotoxicity of doxorubicin and mitoxantrone is due to intercalation into DNA. Although the mechanism of intercalation is not dependent of pH, the influence of mitoxantrone and doxorubicin on topoisomerase II activity is optimal at alkaline pH [9]. Furthermore, cytotoxicity to mitoxantrone is increased at higher pH, since it can complex with iron or copper cations, thereby generating reactive oxygen species (ROS) [19]. The lack of ROS results in reduced DNA damage thereby preventing the cells from apoptosis. This additional effect on the cytotoxicity of mitroxantrone by reduction of ROS under higher pH is not observed for doxorubicin [16]. In tumours, decreased drug accumulation due to lower pH of the microenvironment may be more pronounced, since tumours become acidic not only at hypoxia but also due to high glycolysis rates, called the Warburg effect [26].

In conclusion, the observed resistance of MCF-7 breast cancer cells to mitoxantrone under hypoxia is unrelated to the presence of the three major MDR transporters and can be explained by a combination of reduced cytotoxicity and a lower intracellular drug concentration. These effects are likely not caused by hypoxia alone, but also by the more acidic microenvironment caused by anaerobic glycolysis.

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